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Baboon endogenous virus genome: Molecular cloning and structural characterization of nondefective viral genomes from DNA of a baboon cell strain

(recombinant DNA library/infectious proviruses/DNA sequence determination/genetic map/transfection)

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ABSTRACT **Several heterogeneities in the baboon endogenous virus (BaEV) genomes that are present in the DNA of normal baboon tissues and the baboon cell strain BEF-3 have been described previously. To study these genomes, we cloned BaEV proviruses from BEF-3 cellular DNA into the λ vector Charon 4A. Of the four full-length clones isolated, one was nondefective as determined by transfection. The sequence of a portion of this clone was found to code for amino acids 61–91 in the p30 region of the *gag* gene. This identification allowed us to align the restriction map with the BaEV genetic map. One heterogeneity, a *Bam*HI site 2.4 kilobases (kb) from the proviral 5′ end, was located close to the *gag–pol* junction; another, a *Bam*HI site 1.4 kb from the 5′ end of the genome, corresponded to the *gag* p30 coding sequence for amino acids 32–34; and a third, a *Xho* I site, was near the 3′ end of the *pol* gene. To select the nondefective BaEV genomes from BEF-3 cells, we infected permissive cells with virus produced by BEF-3 cells and also transfected BEF-3 cellular DNA into permissive cells. The BaEV genomes in the permissive recipient cultures were then analyzed by restriction enzyme analysis. These nondefective genomes were found to be heterogeneous with respect to the *gag–pol Bam*HI site and the *Xho* I site, but all were found to contain the *Bam*HI site 1.4 kb from the 5′ end of the genome.**

Baboon cellular DNA contains numerous copies of the baboon endogenous virus (BaEV) genome. Although these sequences are not normally expressed at detectable levels in baboon cells, the cells do reproducibly release infectious BaEV under certain conditions. Thus, it appears that at least some of the viral genomes in baboon cells are complete nondefective proviruses.

In a previous study we analyzed the BaEV genomes in the baboon cell strain BEF-3 by restriction mapping (1). This cell strain is unusual in that it produces high levels of BaEV, it contains even more BaEV genomes per diploid equivalent of DNA than does normal baboon tissue, and, unlike baboon tissue DNA, its cellular DNA gives rise to BaEV upon transfection of permissive cells. In our previous studies we found a considerable degree of sequence heterogeneity among the BaEV genomes in BEF-3 cellular DNA.

As one approach to understanding the significance of these polymorphisms in the BaEV genomes, we have evaluated the infectivity of a number of different genomes isolated from BEF-3 cells. Several strategies were used in the isolation of individual nondefective genomes, including recombinant DNA methods as well as more classical virological techniques. Each isolated genome was characterized by restriction mapping; in addition, the restriction map of one proviral clone was aligned with the BaEV genetic map by DNA sequence analysis of recombinant bacteriophage DNAs containing BaEV genomes. The results of these studies show that: (*i*) only a subset of the BaEV genomes in BEF-3 cells are nondefective; (*ii*) there is sequence heterogeneity even within this subset; and (*iii*) different genomes within this subset differ with respect to the rate of virus replication after transfection of permissive cells.

MATERIALS AND METHODS

Virus Isolation. Filtered supernatant from BEF-3 cells, a baboon embryo fibroblast cell strain that releases BaEV (2), was diluted serially 1:10. We applied 1 ml of each dilution to at least 15 cultures of a human RD cell clone (3). The RD cells were grown in Eagle's minimal essential medium with 10% fetal calf serum. After 4 weeks, the cultures were tested for the presence of retrovirus by reverse transcriptase assay. An end-point dilution was defined as the highest dilution at which at least one culture was producing virus. DNA was isolated, as described (1), from all reverse transcriptase-positive cultures.

Transfection. The D17 line of dog cells (4) was grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum. Sixty-millimeter dishes were seeded with 8×10^5 D17 cells. The following day the cells were exposed to a calcium phosphate-DNA precipitate for 4 hr in growth medium (5) and then treated with 15% glycerol in 0.13 M NaCl/5 mM KCl/20 mM D-glucose/20 mM Hepes, pH 6.9/0.35 mM Na_2HPO_4 (HBS) for 2 min. Transfections were performed with 15 μg of cellular DNA or with 1 μg of clone DNA plus 15 μg of uninfected dog cell (carrier) DNA. Each experiment included a mock-transfected control which was treated with carrier DNA alone; these control cultures were uniformly negative in all virus assays.

Transfected cells were passaged in growth medium with Polybrene (2 μg/ml) for up to 8 weeks. They were screened for BaEV production by *in situ* hybridization of the monolayers with BaEV [^{32}P]cDNA (details to be published elsewhere). All cultures that were positive in this rapid assay ultimately became positive in reverse transcriptase assays as well. The cultures were also tested for BaEV p30 by competition radioimmunoassay (courtesy of C. V. Benton, Frederick Cancer Research Center). The cells that had been exposed to BEF-3 DNA or clone 14A2 DNA were found to contain 2.5–5.3 μg of BaEV p30 protein per mg of cell protein; the mock-transfected cultures contained less than 0.03 μg/mg.

Molecular Cloning, Restriction Mapping, and DNA Sequence Analysis. BEF-3 cell DNA was partially digested with

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Abbreviations: BaEV, baboon endogenous virus; kb, kilobase(s); bp, base pair; R-MuLV, Rauscher murine leukemia virus.

*Eco*RI (New England BioLabs) for 5, 10, 15, or 20 min at 0.1 enzyme unit/μg of DNA. The digests were pooled and centrifuged in sucrose gradients. DNA 15–25 kb long was selected and ligated into the *Eco*RI arms of Charon 4A λ DNA (6). This DNA was then packaged *in vitro* into infectious λ phage (7, 8) to yield a library of BEF-3 cell DNA fragments containing 1.9×10^5 infectious particles (9). After amplification of the library by plate lysis growth in *E. coli* Dp 50 sup F, we screened 1.5×10^5 plaques by filter hybridization (10) with a BaEV [^{32}P]cDNA probe (11). The recombinant clone DNAs and high molecular weight cellular DNAs were mapped by restriction enzyme analysis in Southern blotting experiments as described (1, 11). We utilized the technique of Maxam and Gilbert (12) for DNA sequence analysis of purified recombinant DNA restriction fragments.

RESULTS

We previously reported (11) the physical map of the BaEV genome derived by restriction analysis of BaEV unintegrated linear DNA. We showed that the restriction map of the integrated BaEV genomes of BEF-3 cells and baboon tissues is consistent with the unintegrated DNA map but that the integrated genomes are heterogeneous with respect to several DNA sequence features summarized in Fig. 1 (1). These include *Bam*HI cleavage sites (ZA) and (BC). The presence of a previously described 150-base-pair (bp) insert in *Bam*HI fragment B (1) has been clarified; instead of an insert, some proviruses contain a previously undetected *Bam*HI site, termed (AB*), located about 150 bp from *Bam*HI site (AB) (unpublished data). The map also shows the presence of a heterogeneous *Xho* I cleavage site 5.5 kb from the 5′ end of the genome (data shown below).

Cloning of Cellular Proviral DNA. Because BaEV unintegrated linear DNA and the BaEV proviruses in BEF-3 cells contain a single *Eco*RI cleavage site, we chose to clone the integrated BaEV proviruses by incomplete *Eco*RI cleavage of the BEF-3 cellular DNA, ligation into Charon 4A λ DNA, and *in vitro* packaging. Our initial screening of the BEF-3 cell recombinant DNA library yielded 20 positive clones. Four of these contained full-length BaEV genome inserts. In these four clones, the lengths of the *Eco*RI inserts were determined by electrophoresis of the fragments released by digestion with *Eco*RI. The restriction maps of these four clones are summarized in Fig. 2.

The genomes of clones 7B2 and 2A1 lack the *Bam*HI site (AB*) [genomes of this class were previously described (1) as containing a 150-bp insert]. Clones 14A2 and 15A1, on the other hand, contain the *Bam*HI site (AB*) (Figs. 1 and 2) and, in this regard, they are similar to the majority of other BaEV proviruses in BEF-3 cellular DNA.

Aligning the Genetic Map with the Restriction Map. In order to localize the BaEV heterogeneities in the genetic map, we began to determine DNA sequences of pertinent regions of the BaEV provirus genome. Clone 14A2 DNA was cleaved with

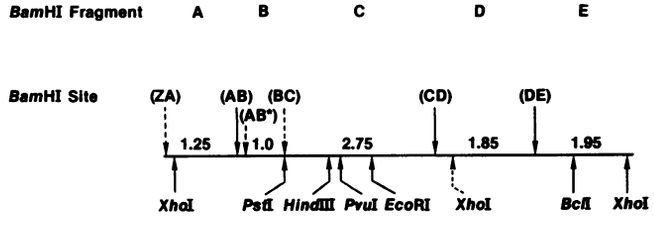

FIG. 1. Restriction map of BaEV genomes in BEF-3 cell DNA. Solid lines, invariant restriction sites in these BaEV genomes; broken lines, observed heterogeneities.

*Bam*HI and the 3.75-kb fragment B + C (Fig. 1) was isolated from an agarose gel after electrophoresis (13). The *Bam*HI ends were labeled with [α-^{32}P]nucleotides by using the Klenow fragment of DNA polymerase (13). After recleavage with *Pst* I and electrophoretic isolation of the 1.0-kb *Bam*HI/*Pst* I fragment, we determined the sequence of a 92-nucleotide-long portion of the fragment by the procedure of Maxam and Gilbert (12) (Fig. 3). As shown below, this region begins 84 nucleotides to the right of *Bam*HI site (AB*) and lies in the *gag* p30 gene. The sequence of the BaEV p30 protein has been determined only through amino acid 36 (14) but that of Rauscher murine leukemia virus (R-MuLV) has been completely determined (ref. 15; L. E. Henderson, T. D. Copeland, and S. Oroszlan, personal communication). When the 92-nucleotide-long BaEV DNA sequence was translated, one reading frame was found to correspond to a run of 29 of 30 identities with the R-MuLV p30 protein, amino acids 61–91. Based on this homology with R-MuLV, we conclude that this sequence is located within the BaEV p30 encoding region.

Having oriented the BaEV *Bam*HI fragment within the *gag* p30 protein, we wanted to determine to which amino acid residues *Bam*HI site (AB*) corresponded. Two results allowed us to do so. First, DNA sequence analysis of an AKR–MuLV recombinant DNA clone (unpublished data) showed that the DNA sequence immediately following a *Bam*HI site codes for a sequence in the *gag* p30 gene beginning at R-MuLV amino acid 35 (Fig. 3). As shown, the region from amino acids 35–72 contains 34 of 38 identities with that of R-MuLV. Furthermore, the AKR–MuLV DNA sequence coding for amino acids 61–72 (a run of 35 nucleotides) is completely identical to the BaEV DNA sequence in this region. Thus, the BaEV genome is homologous not only to R-MuLV but also to AKR–MuLV in the *gag* p30 gene. Second, upstream from the sequenced 92 bp of BaEV DNA, we searched for a R-MuLV amino acid sequence corresponding to one translated reading frame of a *Bam*HI recognition site, 5′ G-G-A-T-C-C 3′. It is not known whether R-MuLV contains a *Bam*HI site in this region but, because amino acid sequence data were available for R-MuLV but not for the closely related AKR–MuLV which does contain a *Bam*HI site in this region (see above), we considered the possibility that the amino acid sequence encoded by the *Bam*HI site is conserved in R-MuLV. Only one such potential *Bam*HI site was located, sequence Glu-Asp-Pro at positions 32–34 in the *gag* p30 protein, and could be encoded by codons GAA/GAG-GAC/*GAT*-*CCN*. In the homologous region of the BaEV p30 protein, the amino acid sequence was reported to be Gln-Asp-(Gln) which could be coded by CAA/CAG-GAC/*GAT*-CAA/CAG (14). However, the third residue, Gln, was only tentatively assigned because proline was also detected in that cleavage step (ref. 14; Fig. 1).

Thus, we conclude that *Bam*HI site (AB*) is part of the coding sequence for the p30 amino acids 32–34, Gln-Asp-Pro, and is located 84 bp to the left of the BaEV fragment that codes for amino acids 61–91. The sequence coding for amino acids 32–34 would be located 96–102 bp from the 5′ end of the p30 part of the *gag* gene, assuming that there is no noncoding DNA in this region. The *gag*-encoded proteins, p30 + p10, total approximately 316 amino acids and thus require 948 bp of the DNA coding sequence. Thus, the p10 gene would terminate ≈850 bp to the right of *Bam*HI site (AB*) or 2.25 kb from the 5′ end of the genome. Because *Bam*HI fragment B is ≈1.0 kb long, this places the heterogeneous *Bam*HI site (BC) ≈150 bp to the right of the end of the *gag* gene.

Infectivity. To determine whether any of the four full-length recombinant clones was nondefective, we assayed their ability to effect the spread of BaEV upon transfection of permissive

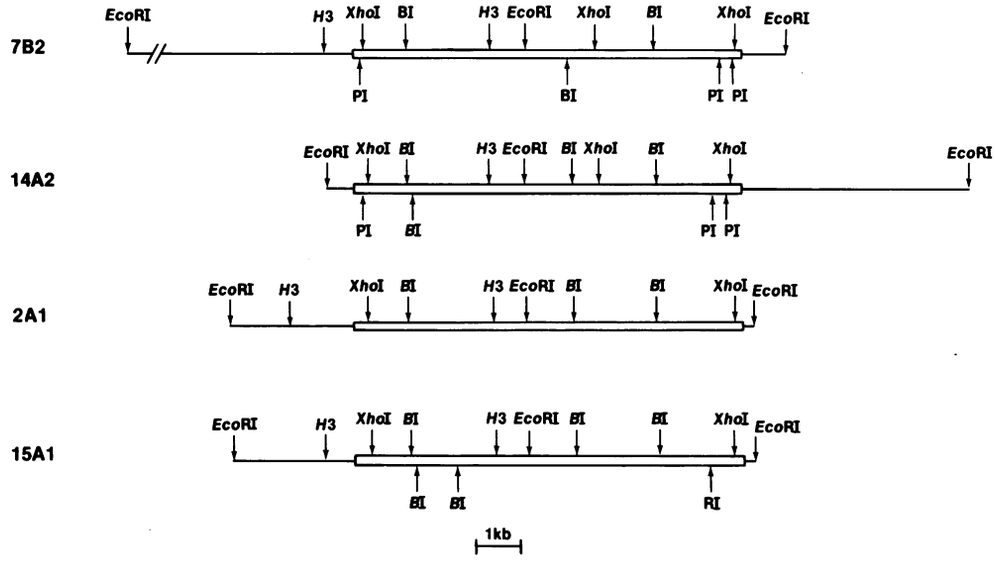

FIG. 2. Restriction maps of four full-length BaEV genomes isolated from the BEF-3 cellular DNA recombinant λ library, showing BaEV genome and baboon flanking DNA restriction sites. *H3*, *Hin*dIII; *BI*, *Bam*HI; *PI*, *Pvu* I.

cells. In repeated tests, clone 14A2 DNA (like BEF-3 DNA) was always positive; clones 7B2, 2A1, and 15A1 were always negative. Cultures transfected with clone 14A2 DNA, however, were reproducibly slower in becoming fully virus-positive than were cultures transfected with BEF-3 DNA, even though the number of genome equivalents of 14A2 DNA used were greater than those of BEF-3 DNA (see below).

Characterization of Infectious Proviruses. Although the DNA of clone 14A2 was infectious, it differed from the unintegrated linear DNA obtained from three independent stocks of BaEV (1, 11) in that it lacked the *Bam*HI cleavage site at (BC). It seemed important to determine whether these differences represented actual genetic differences or reflected some other difference between the two sources of DNA. For example, it seemed possible that genomes lacking the *Bam*HI site rapidly regenerate this site during viral growth in eukaryotic cells or that a DNA modification could obscure this site in DNA isolated from 14A2 bacteriophage particles. To test these possibilities, we transfected clone 14A2 DNA onto dog cells, allowed the progeny BaEV to spread through the dog cell culture, and then examined the integrated BaEV genomes in this culture for the presence of the *Bam*HI site. The proviral DNA in these cells was a faithful replica of 14A2 DNA, lacking *Bam*HI site (BC) (Fig. 4, lanes B and E). The controls in lanes A and C were *Bam*HI digests of BEF-3 DNA and a baboon tissue DNA, S984 (1), respectively. Thus, the BaEV genome in clone 14A2 "breeds true" upon virus passage in mammalian cells; furthermore, the absence of the *Bam*HI site is a stable genetic property by which

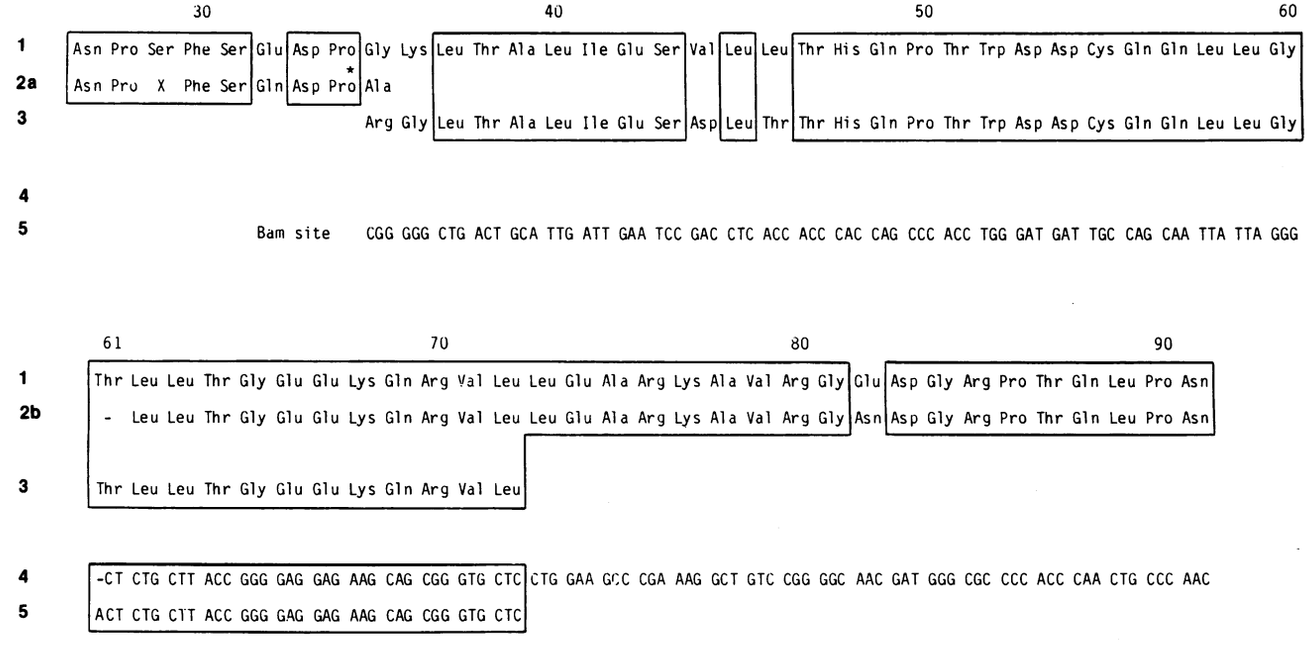

FIG. 3. Schematic representation of DNA sequence and amino acid sequence data from a portion of the *gag* p30 gene of BaEV, R-MuLV, and AKR–MuLV. *, Tentative amino acid assignment (see text). 1, R-MuLV amino acid sequence; 2a, BaEV amino acid sequence; 2b, BaEV, amino acid sequence derived from DNA sequence; 3, AKR amino acid sequence derived from DNA sequence; 4, BaEV DNA sequence; 5, AKR DNA sequence.

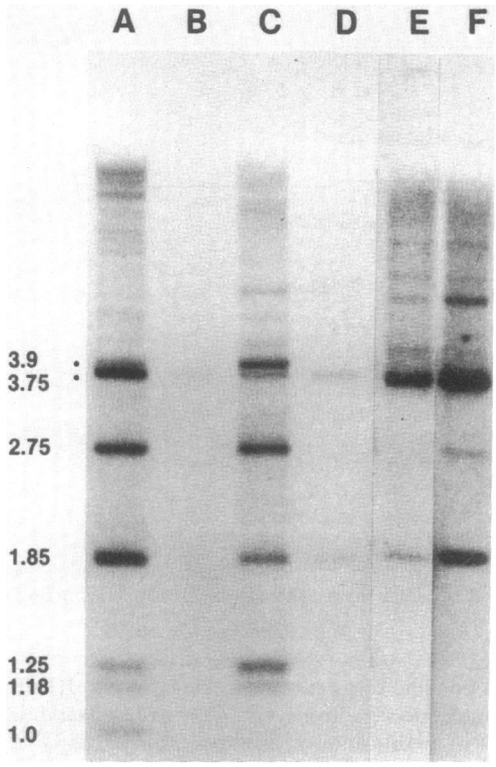

FIG. 4. Hybridization of BaEV [^{32}P]cDNA to the *Bam*HI digests of transfected cellular DNA. Lanes: A, *Bam*HI digest of BEF-3 cellular DNA; B, *Bam*HI digest of DNA from a culture of D17 cells that had been transfected with clone 14A2 DNA; C, *Bam*HI digest of DNA from a baboon tissue, S984 (1); D, *Bam*HI digest of DNA from a culture of D17 cells that had been transfected with BEF-3 DNA; E and F, longer autoradiographic exposures of B and D, respectively.

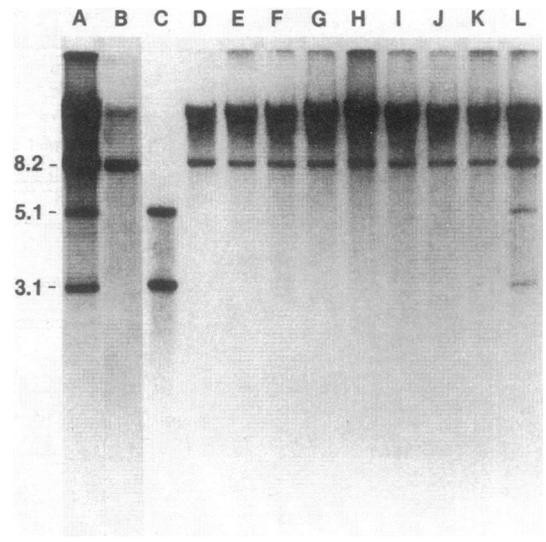

FIG. 5. Differences in *Xho* I restriction sites among individual isolates of BaEV. Lanes A and L, *Xho* I digest of BEF-3 cellular DNA; B, *Xho* I digest of DNA from a culture of D17 cells that had been transfected with BEF-3 DNA; C, *Xho* I digest of clone 14A2 DNA; D–K, *Xho* I digests of DNA from eight cultures of RD cells that had been infected with end-point dilutions of BEF-3 supernatant virus. Lanes A and B are from a different agarose gel than lanes C–L.

it differs from other infectious BaEV genomes.

We also analyzed the infectious BaEV genomes in BEF-3 cells directly. Two separate experimental approaches to this question were used: (*i*) analysis of genomes replicated after transfection of BEF-3 DNA; and (*ii*) analysis of the genomes derived from individual particles of infectious BaEV produced by BEF-3 cells.

In the former, we determined whether the integrated BaEV proviruses in dog cells (the result of BEF-3 cell DNA transfection and virus spread) contained *Bam*HI site (BC) and the internal *Xho* I site (see below). When DNA from the dog cells that had been exposed to BEF-3 DNA was digested with *Bam*HI (Fig. 4, lanes D and F), two classes of fragments were observed: (*i*) intense bands at 3.75 kb due to molecules that were not cleaved at *Bam*HI site (BC) and at 1.85 kb, the other internal *Bam*HI fragment; and (*ii*) faint bands at 2.75 and 1.0 kb due to molecules that were cleaved at *Bam*HI site (BC). Densitometric tracings (not shown) of this autoradiogram indicated that the 2.75-kb-band intensity was about 0.12 of that expected for a fragment of that length if it were present in equal molar proportion to the 1.85-kb band. Thus, BEF-3 DNA does contain two classes of infectious proviruses that differ in the presence of *Bam*HI site (BC).

Some of the BaEV genomes in BEF-3 DNA were cleaved by *Xho* I to yield a 5.1- and a 3.1-kb fragment (Fig. 5, lanes A and L). Unlike the case with *Bam*HI, no *Xho* I site heterogeneity was seen in the integrated BaEV genomes after transfection of BEF-3 DNA onto dog cells. All BaEV proviruses that were detected lacked the *Xho* I site (Fig. 5, lane B).

The second experimental approach for studying the infectious BaEV genomes in BEF-3 cell DNA was through analysis of newly integrated proviruses in the DNA of RD cell cultures individually infected with single infectious BaEV particles. DNA from 14 end-point-positive cultures was digested with *Bam*HI. The results paralleled those observed in dog cells after transfection of BEF-3 DNA. DNA from 10 cultures revealed integrated BaEV genomes lacking the *Bam*HI site (BC), from 3 revealed genomes having the *Bam*HI site, and from 1 (lane B) revealed genomes of both types (Fig. 6). Also consistent with the transfection results was our observation that 13 of 14 end-point-culture DNAs revealed BaEV genomes lacking the internal *Xho* I restriction site (8 are shown in Fig. 5). One DNA (Fig. 5, lane D) revealed not only the 8.2-kb *Xho* I fragment but, after long autoradiographic exposure (not shown), also revealed trace amounts of the 5.1- and 3.1-kb *Xho* I fragments as well. We interpret this to mean that this culture (which is a different culture from the one described above as having two BaEV genomes with different *Bam*HI sites) was either infected with two

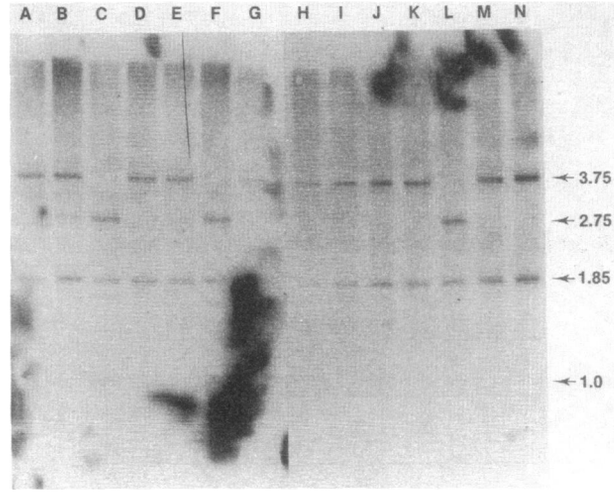

FIG. 6. *Bam*HI digests of DNA from 14 cultures of RD cells that had been infected with end-point dilutions of BEF-3 supernatant virus.

virus particles that differed with respect to the internal *Xho* I site or that a mutation at that site occurred during growth in culture.

DISCUSSION

We have previously shown that the BaEV genomes in BEF-3 cells and baboon tissues are quite heterogeneous (1). The present report extends this work and focuses on those viral genomes in BEF-3 cells that are infectious. As a first step in characterizing these heterogeneities, we prepared a library of BEF-3 DNA fragments cloned into the λ vector Charon 4A and isolated several recombinant DNA clones containing full-length BaEV proviral inserts. One of the full-length clones, clone 14A2, was found to be infectious for dog cells.

We next attempted to localize the observed provirus heterogeneities in the BaEV genetic map. By sequencing 92 nucleotides in the DNA of clone 14A2, we were able to align the DNA restriction map precisely with a known protein sequence in the *gag* p30 gene (Fig. 3). Thus, we mapped one of the earlier reported BEF-3 provirus heterogeneities, *Bam*HI site (BC). If there is no noncoding DNA in the *gag* gene or between the *gag* p10 and the polymerase genes, *Bam*HI site (BC) is located in the polymerase gene near its 5′ end. Furthermore, *Bam*HI site (AB*) was found to be part of the *gag* p30 coding sequence for amino acids 32–34. Recent unpublished DNA sequence data in the *env* gene region allowed placement of the internal *Xho* I site within the 3′ end of the *pol* gene.

As we have shown, only a subset of the BaEV proviruses in BEF-3 cells have any detectable infectivity. In particular, two classes of BEF-3 proviruses are conspicuously absent in the infectious provirus population isolated by either recombinant or virological techniques: (*i*) genomes lacking *Bam*HI site (AB*); and (*ii*) those containing *Bam*HI site [termed (ZA)] near the 5′ proviral end.

The nondefective genomes of BEF-3 cells are, themselves, heterogeneous. The single infectious clone that we isolated (clone 14A2) does not have *Bam*HI site (BC) in its genome but does contain the internal *Xho* I site. On the other hand, the proviruses detected in permissive cell DNA after infection of individual BaEV particles or transfection of BEF-3 cellular DNA may or may not contain *Bam*HI site (BC) but for the most part do not contain the *Xho* I site.

The nondefective BaEV proviruses are heterogeneous in their ability to replicate in permissive cells. When tested for infectivity, cultures transfected with clone 14A2 DNA were reproducibly slower in becoming fully virus-positive than were cultures transfected with BEF-3 DNA. That is, 1 μg of clone 14A2 DNA containing ≈195 ng of BaEV proviral DNA resulted in slower infection than did 15 μg of BEF-3 cell DNA containing ≈3 ng of BaEV proviral DNA, which consists of ≈100 proviral genomes. Even if BEF-3 cellular DNA contains 10 infectious proviruses per diploid equivalent, more than 650 times as much clone 14A2 proviral DNA as BaEV sequences in these BEF-3 proviruses resulted in a weaker transfection after 3 weeks in culture. This result, together with the fact that no infectious proviruses containing the internal *Xho* I site were detected in permissive cell DNA after transfection with BEF-3 DNA or infection with individual BaEV particles [except for traces in one end-point culture (Fig. 5)] indicates that the *Xho* I site-containing proviruses in BEF-3 cellular DNA either replicate more slowly than do other BaEV proviruses or that most of these genomes are not infectious.

Thus, the proviruses that transfection and infection experiments detected are those that replicate best (even though others may also replicate, as shown for clone 14A2). Also, the absence of infectious genomes of a particular class [e.g., those lacking *Bam*HI site (AB*)] does not necessarily mean that the feature is lethal but just that it is not present in the infectious genomes that we detected.

The above results show that the nondefective proviruses of BEF-3 cells may or may not contain *Bam*HI site (BC) which is located near the 5′ end of the *pol* gene; however, a *Xho* I site near the 3′ end of the *pol* gene of one nondefective BaEV clone (14A2) is not present in the other nondefective BaEV genomes that we detected.

Note Added in Proof. It might be suggested that the failure of DNA from dog cells transfected with BEF-3 DNA to be cleaved internally by *Xho* I (Fig. 5, lane B) is because this site is methylated in dog cells. However, we have now found that the proviruses resulting from transfection of clone 14A2 into dog cells are largely cut at this position by *Xho* I. Therefore, methylation of the site does not explain the failure of BEF-3 transfected dog DNA to be cleaved there.

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